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Micro-arc oxidation as a tool to develop multifunctional calcium-rich surfaces for dental implant applications

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ABSTRACT

Titanium (Ti) is commonly used in dental implant applications. Surface modification strategies are being followed in last years in order to build Ti oxide-based surfaces that can fulfill, simultaneously, the following requirements: induced cell attachment and adhesion, while providing a superior corrosion and tribocorrosion performance. In this work micro-arc oxidation (MAO) was used as a tool for the growth of a nanostructured bioactive titanium oxide layer aimed to enhance cell attachment and adhesion for dental implant applications. Characterization of the surfaces was performed, in terms of morphology, topography, chemical composition and crystalline structure. Primary human osteoblast adhesion on the developed surfaces was investigated in detail by electronic and atomic force microscopy as well as immunocytochemistry. Also an investigation on the early cytokine production was performed. Results show that a relatively thick hybrid and graded oxide layer was produced on the Ti surface, being constituted by a mixture of anatase, rutile and amorphous phases where calcium (Ca) and phosphorous (P) were incorporated. An outermost nanometric-thick amorphous oxide layer rich in Ca was present in the film. This amorphous layer, rich in Ca, improved fibroblast viability and metabolic activity as well as osteoblast adhesion. High-resolution techniques allowed to understand that osteoblasts adhered less in the crystalline-rich regions while they preferentially adhere and spread over in the Ca-rich amorphous oxide layer. Also, these surfaces induce higher amounts of IFN-γ cytokine secretion, which is known to regulate inflammatory responses, bone microarchitecture as well as cytoskeleton reorganization and cellular spreading. These surfaces are promising in the context of dental implants, since they might lead to faster osseointegration.

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1. Introduction

Titanium is successfully used in dental implants due to their excellent biocompatibility and mechanical properties when implanted in the jaw. Despite the high success of titanium implants, the shortening of the oral rehabilitation time, without compromising osseointegration, still is a topic of particular interest in dentistry, due to its influence on the quality of life of patients. “Active” surfaces have been developed aiming to accelerate osteogenesis in order to allow implants loading within 1 week subsequent to implant placement [1]. It has been reported that osseointegration rate is dependent on surface composition and

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roughness, where rough implants favor both bone anchoring and biomechanical stability.

Osteoconductive calcium phosphate coatings stimulate bone healing, leading to the rapid fixation of the implants [2–6]. However, the bonding between the implant and bone tissue is not always satisfactory, while the adhesion of those coatings to the titanium substrate is a matter of concern [7,8]. Surface modifications able to change surface energy, chemistry or topography to improve the early bone-to-implant response and guarantee a favorable outcome of the implant are being reported in the recent literature [2–4]. Among different surface functionalization techniques, special attention has been given to micro-arc oxidation which may promote an increase of surface bioactivity through the manipulation of the chemistry of the native TiO_2 layer by the incorporation of ionic species, such as calcium (Ca), phosphorus (P) and magnesium (Mg), elements natively present in the bone [9–16]. It is already known that anodized titanium doped with Ca and P ions interacts better with the surrounding bone and directly affects cellular responses such as osteoblast proliferation, differentiation, gene expression as well as the overall osseointegration process [9,13,14,16–22]. Santos et al. produced and characterized porous titanium oxide layers produced by anodic treatments and observed that those surfaces were hydrophilic without revealing any kind of cytotoxicity [23]. H. P. Felgueiras et al. recently demonstrated that these anodic surfaces enhance osteoblast attachment, differentiation (ALP production and mineralization) as well as osteointegration [16]. In fact also clinical observations have shown that oxidized implants demonstrate stronger bone anchorage comparing to machined implants in both animal and human experiments [6,8,24]. Cell–surface interactions are also mediated at a molecular level [25,30]. It has been shown that pre-osteoblast and fibroblast adhesion is influenced by the atomic structure of the surface titanium oxide crystals due to the interaction between the functional group of extracellular matrix proteins and the atomic order of the surface of these crystals [31–35]. Although several theories have been proposed, evidence of the effect of the TiO_2 crystal structure on its biocompatibility remains inconclusive. While some works indicate an enhanced cell behavior to anatase [4,36–38], others refer that rutile promotes a better osseointegration [34,35,39]. It is known that rutile has higher chemical stability comparing to anatase, exhibiting a superior apatite-forming ability, *i.e.* bioactivity [35,39]. The interaction of hepatocytes and osteoblasts with rutile was already reported by some authors [32,38]. On rutile-rich films produced on β-Ti alloys by the micro-arc oxidation technique, an improved osteogenesis performance was observed [34,40]. The crystallographic lattice matching of rutile with that of apatite was referred as a possible mechanism for this behavior, resulting in an excellent apatite-forming ability of rutile surfaces [33,41]. Differential affinities of macromolecules and proteins to the different TiO_2 crystallographic phases were also reported as possible mechanisms for that behavior. Still regarding the crystalline structure, some works highlight the role of anodic treatments, as a method to obtain rutile-rich surfaces for better tribocorrosion behavior, thus minimizing the degradation of the material [9,42–44]. In fact, Alves et al. [9] demonstrated the important role of rutile in the improvement of the tribocorrosion behavior of the surfaces being used in this work.

In this work MAO was used as a tool to develop a new multiphasic nanostructured titanium oxide film with graded structure (anatase, rutile and amorphous phases) and chemical composition (essentially Ca). The main aim of this paper was to investigate bone derived cell attachment and adhesion to those surfaces. These initial cell events occurring on implant surfaces are prerequisites for the success of osseointegration and bone regeneration process. As a proof of concept the *in vitro* adhesion mechanisms of primary human osteoblasts on these surfaces were investigated using electronic microscopy (SEM and TEM), coupled atomic force/Raman microscopy and immunofluorescence techniques. A study of the early gene and cytokine expression was also carried out, since not much research has been focused on gene and cytokine regulation by anodic treated surfaces.

2. Experimental section

2.1. Preparation and characterization of multifunctional titanium surfaces

A sheet of commercially pure titanium (Ti) grade 2 (Goodfellow Cambridge Ltd, UK) with 2 mm thickness was cut into squares of 10×10 mm and consecutively cleaned in acetone for 3 min and finally warm air-dried. Prior to anodic treatment, all the specimens were etched in a Kroll's reagent solution (2% HF, and 10% HNO_3, in 88% H_2O) for 10 min in order to remove the native oxide layer. These substrates were ultrasonically cleaned in propanol and water, and dried with warm air. The etched samples (Ti) were used as control samples. The electrolyte used for the anodic treatment was a solution of 0.02 M β-glycerophosphate disodium salt pentahydrate (β-GP) (Fluka-BioChemika) and 0.35 M calcium acetate monohydrate (CA) (Sigma-Aldrich), β-GP and CA being used as P and Ca sources, respectively. The anodic treatment was performed at room temperature using a dc power supply (GPR-30H10D). This treatment was carried out for 1 min at a constant voltage of 300 V [8].

A detailed surface characterization was performed on both surfaces (Ti and Ti CaP). The surface morphologies and chemical composition analysis of Ti and Ti CaP were studied with a field emission scanning electron microscope (FEI Magellan 400) equipped with an energy dispersive X-ray analysis unit (EDX). The structure and topography of both surfaces were analyzed by the means of a coupled atomic force microscope/confocal Raman (Witec Alpha300 AR). The surface characterization was performed in 3 to 5 different specimens, depending on the characterization technique. Prior to cell culture tests, all disks were

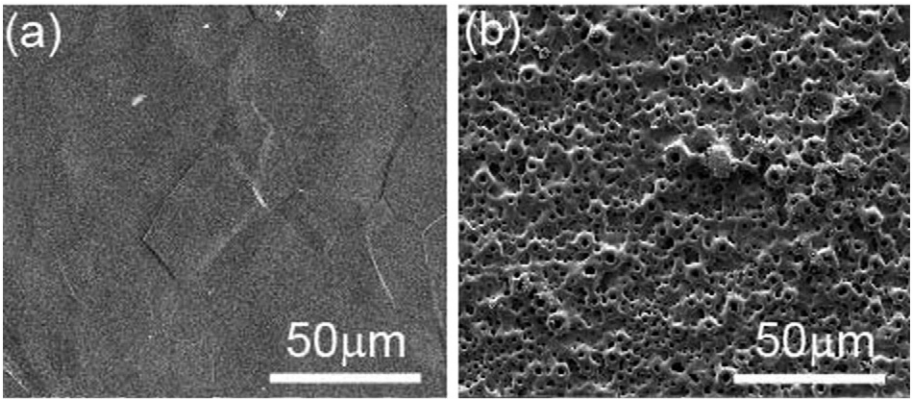

Fig. 1. Morphology of Ti surfaces: SEM micrographs showing Ti (a) and TiCaP (b) surface morphology.